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Activated Charcoal Diminishes the Lot Difference of Fetal Bovine Sera in Erythroid Colony Formation of Human Bone Marrow Cells (42781)

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Abstract. Using normal bone marrow as target cells, we assayed the colony-forming efficiency of early and late erythroid progenitor cells and granulocyte-macrophage progenitor cells using several different lots of fetal bovine serum (FBS). There was a marked difference in the ability of these sera to support colony formation, particularly in erythroid colony assays. When adsorbed by activated charcoal, all these sera supported erythroid colony formation more efficiently than before adsorption. There was no significant effect of charcoal adsorption of FBS on granulocyte–macrophage colony formation. Gel-filtration study showed that charcoal adsorption diminished low-molecular-weight fractions by less than 5000 Da. The inhibitory activity of this fraction was heat labile and Pronase sensitive. Concentrated samples obtained from these fractions inhibited erythroid colony formation in a dose-dependent manner. These results suggest that low-molecular-weight inhibitors that are relatively specific to erythropoiesis play a critical role in the lot differences of FBS for erythroid colony formation.

There are substantial differences in colony formation by hemopoietic precursor cells in clonogenic culture with various lots of fetal bovine serum (FBS). These differences exist even when sufficient amounts of colony-stimulating factors, such as burst-promoting activity (BPA) and erythropoietin (EPO) for erythropoiesis or colony-stimulating factor (CSF) for granulocyte–macrophage lineage, are added. Several possibilities may account for these differences. First, some nutrients essential for cell growth or unknown colony-stimulating factors other than BPA, EPO, or CSF may be deficient in certain sera. Another possibility is the presence of colony inhibitory activities in the sera. Several reports have suggested the presence of erythropoiesis-inhibiting factors in uremic sera (1–5). However, there has been much discussion concerning which of the nutrients, hormones, and/or inhibitors present in serum regulates cell growth *in vitro.* Recently, Lindquist and De-Alarcon (6) reported the removal of inhibitors of human CFU-Meg from fetal bovine serum by charcoal–dextran treatment, but their inhibitors have not been molecularly identified to date. This method originated in the report using charcoal coated with albumin or dextran to separate free hormones such as insulin from protein-bound ones (7).

With charcoal treatment, this study aimed to determine whether serum lot differences in hemopoietic colony assays are due mainly to the deficiency of some unknown stimulating factors or to the presence of inhibitory factors in given sera. The properties of erythroid inhibitory factor removed from serum with charcoal adsorption were analyzed to compare it with various defined inhibitory substances previously described.

Materials and Methods. *Sera.* Eight different lots of fetal bovine sera were purchased from Boehringer-Mannheim (West Germany), GIBCO (NY, USA), and Flow Laboratories (VA, USA) and heat-inactivated at 56°C for 30 min to avoid nonspecific complement-dependent cytotoxicity.

Activated charcoal adsorption. Sera or fractionated eluates were incubated with activated charcoal (Sigma, USA) at a concentration of 12.5 mg/ml for 30 min with agitation and then centrifuged at 2000*g*. Supernatants were sterilized with a 0.45-μm Millipore filter.

Gel filtration of sera. Sera were chromatographed on a Bio-Gel A5m column (3 × 60 cm) equilibrated with 0.05 M NH_4HCO_3.

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Ten milliliters of serum was applied and 10 ml of each elution was collected. Samples of each fraction were lyophilized and reconstituted with α-medium at 15-fold concentration.

Heat stability test. Fractionated samples obtained from gel filtration of FBS were heated at 70, 80, or 100°C for 15 min and cooled at room temperature.

Molecular weight exclusion test. In order to characterize the molecular size, the fractionated samples were applied to Amicon Diaflo YM-2 and YM-10 membranes (exclusion limit, 1000 and 10,000 Da, respectively, Amicon Corp., MA, USA). After filtration, samples were lyophilized. They were reconstituted with an equal volume of water just before use.

Proteolytic digestion. Proteolytic digestion was performed as previously described (8). Fractionated samples obtained with gel filtration of FBS were incubated with Pronase solution (1 mg/ml) (Kaken Chemical Co., Ltd., Tokyo, Japan) at 37°C for 15 hr. This digest was then dialyzed against water with the use of a 1000 Da exclusion-limit membrane (Spectrapor 6 cellulose dialysis tube, Spectrum Medical Industries Inc., CA, USA) and lyophilized until use.

Bone marrow cells. Bone marrow was obtained from normal volunteers with their informed consent. A protocol for obtaining bone marrow was reviewed by the Human Experiment Committee of the First Department of Internal Medicine, Nagoya University School of Medicine. Nonphagocytic mononuclear cells were collected by density centrifugation (400*g*, 30 min) over Ficoll–Paque (Pharmacia, Piscataway, NJ) after incubation with silica particles (KAC-2, Otsuka Assay Lab., Tokyo, Japan) at 37°C for 60 min with intermittent agitation.

Bone marrow culture techniques for BFU-E, CFU-E, and CFU-GM assays. Erythroid burst- and colony-forming units (BFU-E and CFU-E) were cultured according to Iscove (9) with minor modifications. Briefly, 10^5 cells were suspended in 1 ml of α-medium containing 30% FBS, 2 U/ml of purified erythropoietin (Toyobo, Osaka, Japan), 1% detoxified BSA (only for BFU-E), and 0.3% agar and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 14 days for BFU-E and 7 days for CFU-E assay.

Colonies consisting of more than 100 benzidine-positive cells were counted as BFU-E and those with more than 8 cells were counted as CFU-E. The culture conditions used for granulocyte–macrophage colony-forming units (CFU-GM) were identical to those for CFU-E except that 150 U/ml of colony-stimulating factor (Chugai Pharm. Co., Tokyo, Japan) was substituted for erythropoietin. Colonies of more than 100 cells were counted at 10 days of incubation.

Results. Table I shows the colony-forming efficiency of BFU-E and CFU-GM of normal bone marrow cells using 8 different lots of fetal bovine serum and the effects of acti-

TABLE I. DIFFERENCE IN COLONY-FORMING EFFICIENCY AMONG VARIOUS LOTS OF FETAL BOVINE SERUM AND EFFECT OF CHARCOAL ADSORPTION (CA)

	BFU-E colonies[a]/10^5 cells			GFU-GM colonies[a]/10^5 cells		
FBS lot	CA (−)	CA (+)[b]	Ratio	CA (−)	CA (+)[b]	Ratio
1	29.7	64.3	2.2	176	188	1.1
2	50.0	86.7	1.7	134	151	1.1
3	20.0	88.2	4.4	142	153	1.1
4	7.0	42.0	6.0	128	127	1.0
5	7.7	30.0	3.9	157	152	1.0
6	3.3	40.3	12.1	170	188	1.1
7	3.7	32.3	8.8	163	166	1.0
8	4.7	29.0	6.2	141	146	1.0

[a] Colony numbers are expressed as mean/10^5 normal bone marrow cells from triplicate cultures.

[b] Test sera were incubated with 12.5 mg/ml of activated charcoal for 30 min and sterilized with a 0.45-μm Millipore filter.

vated charcoal adsorption of these sera. The colony-supporting ability of FBS varied from lot to lot in BFU-E colony formation but not in CFU-GM colony formation. Charcoal adsorption enhanced the BFU-E colony-supporting ability of all these FBS and diminished the lot difference. This treatment did not affect the CFU-GM colony-supporting ability of FBS.

Figure 1 shows an elution profile of FBS and that of FBS adsorbed with activated charcoal through a Bio-Gel A5m column. Fractions of molecular weight less than ca. 5000 were completely depleted in adsorbed sera, whereas the remaining elution profiles were essentially the same before and after adsorption. These eluates were pooled into three fractions: a low-molecular weight-fraction (Fr 1), a middle-molecular-weight fraction (Fr 3), and a high-molecular-weight fraction (Fr 2). Samples of each fraction were pooled and concentrated 15-fold. Half of these three fractions were adsorbed with activated charcoal. Twenty microliters of both pre- and postadsorbed fractions was added to optimal cultures for BFU-E assay prepared with charcoal-adsorbed FBS (Fig. 2). The unadsorbed sample of Fr 1 inhibited colony growth of BFU-E, and this inhibitory activity was diminished by adsorption with activated charcoal. No other inhibitory activity was found in the remaining fractions.

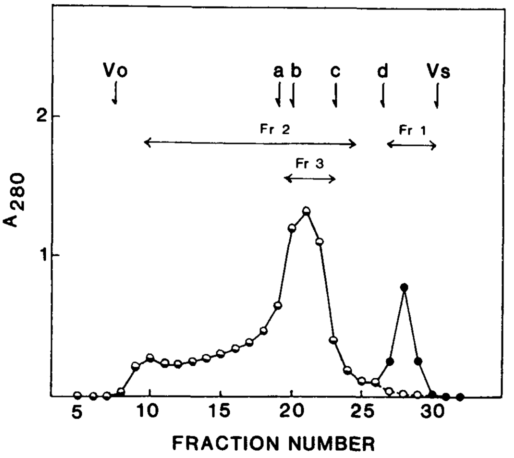

FIG. 1. Gel-filtration profile of FBS on a Bio-Gel A5m column. The solid circles denote an elution pattern of FBS and the open circles denote that of FBS adsorbed with activated charcoal. V_0, void volume; a, bovine transferrin (76,000); b, bovine serum albumin (66,000); c, bovine carbonic anhydrase (29,000); d, bovine aprotinin (6500); V_s, salt volume. Vertical axis denotes uv absorbance at 280 nm.

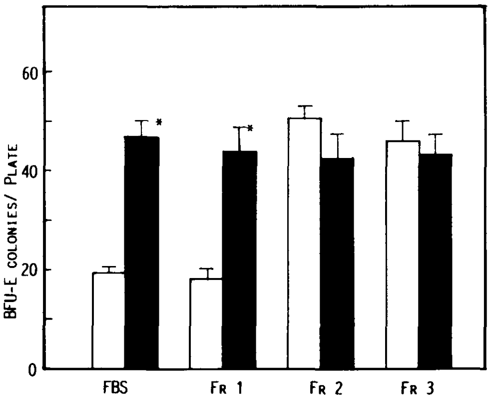

FIG. 2. BFU-E colony inhibitory activity of FBS samples separated by gel filtration. The open column of FBS denotes BFU-E cultured with FBS. The solid column of FBS denotes BFU-E cultured with charcoal-adsorbed FBS. Each fraction (Fr 1, 2, and 3) was added to BFU-E cultures prepared with charcoal-adsorbed FBS. The open columns of Fr 1, 2, and 3 denote addition of each fraction from Fig. 1 to BFU-E cultures prepared with charcoal-adsorbed FBS. The solid columns denote addition of charcoal-adsorbed fractions. Final concentration of FBS for BFU-E assay was 30%. The quantity of the added fraction (20 μl/ml of 15-fold concentrated sample) is the same amount present in original nontreated FBS. *Statistically significant ($P < 0.01$) between the open and the solid columns with t test.

Figure 3 shows the relationship between the dose of concentrated fraction (Fr 1) added to cultures and the number of BFU-E colonies grown under the optimal culture conditions using charcoal-adsorbed FBS. Colony formation declined in a dose-dependent manner as the inhibitory fraction was increased. Twenty microliters of 15-fold concentrated Fr 1 added to the optimal culture prepared with 30% adsorbed FBS is supposed to be the equivalent amount of factors present in 30% nonadsorbed FBS.

A partial characterization of the BFU-E colony-inhibitory activity of fraction 1 is shown in Table II. The inhibitory activity was diminished by heating at 80°C for 15 min and by proteolytic digestion. By molecular weight exclusion, it appears that the inhibitory activity ranges between 1000 and 10,000 Da.

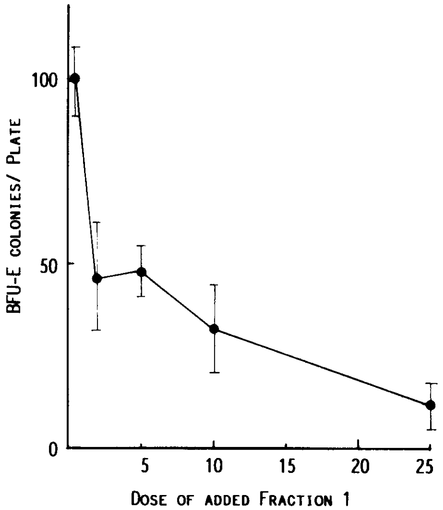

FIG. 3. Dose–response curve of BFU-E colony inhibition by Fr 1. Eluates of Fr 1 were pooled and concentrated 15-fold and added to BFU-E cultures prepared with charcoal-adsorbed FBS. Abscissa denotes microliters of concentrated Fr 1 per culture.

Discussion. Charcoal has been used to remove growth factors and hormones from serum (10). Among the factors removed were T3, T4, insulin, estradiol, testosterone, DGF, and IGF-1. Because glucocorticoid is known to inhibit erythroid colony formation *in vitro* (11), it is a possible erythropoiesis inhibitory factor in the serum. However, Lindquist and De Alarcon (6) reported that there was no significant difference in the concentrations of endotoxin, cortisol, erythropoietin, T3, T4, insulin, estradiol, and testosterone before and after charcoal–dextran treatment of FBS. In our preliminary study, the cortisol levels of our FBS lots measured by RIA were less than 2.5×10^{-8} *M*, and no significant change was observed after charcoal adsorption. Addition of cortisol equivalent to FBS to the optimal cultures for BFU-E assay prepared with charcoal-adsorbed serum did not inhibit colony formation compared with our inhibitory fraction (data not shown). Thus, cortisol is not likely to be the major erythroid inhibitory activity in our system.

According to the elution profile, it is less likely that charcoal adsorption could produce new factors to stimulate erythroid progenitor cells. The profile eliminated erythropoiesis inhibitory molecules of less than about 5000 and greater than 1000 Da. The adsorption experiment of Fr 1 shown in Fig. 2 confirmed that Fr 1 is identical to essential parts adsorbed with charcoal from FBS. A molecular weight exclusion experiment using Amicon Diaflo membranes YM2 and YM10 confirmed the molecular range of the inhibitory activity. The final concentration of Fr 1 necessary to inhibit 50% of BFU-E colony formation in the experiment of Fig. 3 is ca. 1–3 μg/ml, although the inhibitory molecule has not yet been purified from Fr 1. However, it is important that this erythropoiesis inhibitory activity is effective in levels present in original nontreated serum as shown in Fig. 3. This activity was also observed in various types of sera (calf, horse, and even in normal human; data not shown). This observation is consistent with a report demonstrating the presence of colony inhibitory factor in normal serum (12). The pres-

TABLE II. CHARACTERIZATION OF BFU-E INHIBITORY FRACTION IN FETAL BOVINE SERUM

Treatment	BFU-E colonies[a]/10^5 cells	% of Inhibition
Charcoal adsorption	118.0 ± 36.6	0
IF[b]	17.3 ± 6.7	85.3
Heating[c]		
70°C for 15 min	41.7 ± 5.5	64.7
80°C for 15 min	102.0 ± 8.1	13.6
100°C for 15 min	89.8 ± 7.5	23.9
Filtration[d]		
mol wt < 1000	104.0 ± 26.8	11.9
mol wt > 1000	16.0 ± 3.6	86.4
mol wt < 10,000	13.3 ± 1.5	88.7
Pronase digestion[e]	83.3 ± 5.8	29.4

[a] Colony numbers are expressed as means ± SD of triplicate cultures.

[b] IF (20 μl/ml of 15-fold concentrated sample) was added to BFU-E culture prepared with charcoal adsorbed serum.

[c] Experimental design was the same as that for IF[b] except that IF was heat-treated.

[d] Molecular weight exclusion was done according to Materials and Methods. Mol wt < 1000 denotes filtrates of IF through YM2 membrane. Mol wt > 1000 denotes the retentate. Mol wt < 10,000 denotes filtrates of IF through YM10 membrane. Each sample was added to BFU-E culture prepared with charcoal-adsorbed serum.

[e] IF was incubated with 1 mg/ml of Pronase at 37°C for 15 hr and dialysed against water. It was added to BFU-E culture prepared with charcoal-adsorbed FBS.

ence of this erythropoiesis inhibitory activity among normal sera from various species suggests some physiological role in the regulation of hemopoiesis.

Various types of inhibitors have been observed during use *in vitro* colony assays. These include higher molecular weight, serum lipoprotein inhibitors that tend to diminish granulocyte–macrophage colony formation (13), lactoferrins (14), acidic isoferritins (15), various monokines such as tumor necrosis factors (16), tumor growth factor-β (17), interferons (16), low-molecular-weight tissue-derived inhibitors such as granulocyte chalone (18), and certain low-molecular-weight inhibitors such as prostaglandins (14) and cortisol (11). However, the significance of these factors in normal or pathological sera remains unclear.

The inhibitory activity identified here was derived from normal sera from various species and had the characteristics of a peptide in the molecular weight range between 1000 and 5000 Da. Most of the factors identified before are not likely candidates, because they were more than 10,000 Da, and some were even less than 1000 Da, and the spectrum of their activity inhibiting each hemopoietic progenitor cells was different from ours. We reported previously the selective inhibition of erythropoiesis by sera or hemodialysate from patients with chronic renal failure (4, 8). It is very interesting that the properties of the inhibitory activity under study here (such as molecular weight range and protease sensitivity) are similar to those observed in uremic sera or hemodialysate. The molecular weight, for example, is different from that of polyamines, which Fisher *et al.* (19) have stressed as erythropoiesis inhibitory factors in chronic renal failure.

Table I shows that the erythropoiesis inhibitory activity affected BFU-E but not granulocyte–macrophage colony formation. It also inhibited CFU-E (data not shown). Serum lot differences in erythroid colony formation were diminished after charcoal adsorption, but some were observed even after charcoal adsorption, suggesting that this inhibitory activity does not act solely to modulate erythroid colony formation.

Taken together, the results of the present study confirmed a marked difference in erythroid colony-forming efficiency, especially in BFU-E, among various lots of fetal bovine serum, suggesting that the lot difference is mainly but not entirely related to an erythropoiesis inhibitor present in FBS. This inhibitory activity which can be removed with charcoal has the characteristic of a heat-labile peptide with a molecular weight between 1000 and 5000 Da. Thus it seems to be different from the factors described to date.

Further purification of this factor(s) is under way in our laboratory.

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